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Letter

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Targeting Tumor Associated Carbonic Anhydrases IX and XII: Highly Isozyme Selective Coumarin and Psoralen Inhibitors

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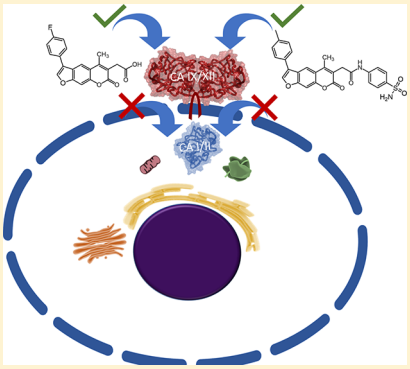
Supporting Information

ABSTRACT: A small library of psoralen carboxylic acids and their corresponding benzenesulfonamide derivatives were designed and synthesized to evaluate their activity and selectivity toward tumor associated human carbonic anhydrase (hCA) isoforms IX and XII. Both psoralen acids and sulfonamides exhibited potent inhibition of IX and XII isozymes in the nanomolar concentration range. However, psoralen acids resulted as the most selective in comparison with the corresponding benzenesulfonamide derivatives. Our data indicate that the psoralen scaffold is a promising starting point for the design of highly selective tumor associated hCA inhibitors.



KEYWORDS: *hCA IX, hCA XII, inhibitors, tumor, coumarin, benzenesulfonamide*

Coumarins are a class of heterocyclic compounds widely distributed in nature with a wide variety of biological activity such as antiviral,[1,2] glucose-lowering agents,[3,4] antimycotic,[5] and antitumor.[6−10]

Calanolides A and B extracted from *Calophyllum lanigerum* show anti-HIV-1 activity.[11] Furthermore, imperatorin has been reported to have anti-inflammatory, antibacterial, antifungal, antiviral, and anticancer activity and might have future clinical application.[12] Noteworthy, other derivatives such as psoralen, bergapten, marmesin, and rutaretin are known antitubercular agents.[13] In particular the 7*H*-furo[3,2-*g*]chromen-7-one derivatives, commonly known as psoralens, have been investigated for their ability to suppress proliferation and angiogenesis in human colon cancer cells by targeting HIF-1α via the mTOR/p70S6*K*/4E-BP1 and MAPK pathways[7] and to reverse multidrug resistance in both lung cancer A549/D16 and breast cancer MCF-7/ADR cells.[14,15]

Moreover, the ability of coumarin derivatives to inhibit human carbonic anhydrase isozymes IX and XII (hCA IX and XII) has also been reported.[16−19]

Noteworthy, the chemical accessibility and the ease of functionalization of the coumarin nucleus allows for synthesizing diverse compound libraries.

The trans-membrane hCA IX and hCA XII isoforms have been associated with tumor progression and invasion.[20−28] In normal conditions, CA IX is expressed in the stomach and few other tissues, while it is ectopically induced and highly overexpressed in many hypoxic solid tumors, by a direct transcriptional activation of the *CA9* gene via HIF-1α. Therefore, the design of new and isozyme selective hCA inhibitors is an attractive challenge for medicinal chemists.

In this respect, the design of new psoralen derivatives as hCA IX and XII inhibitors could be advantageous and may lead to the identification of multitarget agents since other cancer related enzymes and pathways could also be hit by such derivatives.[6,7,9,14,15,29,30]

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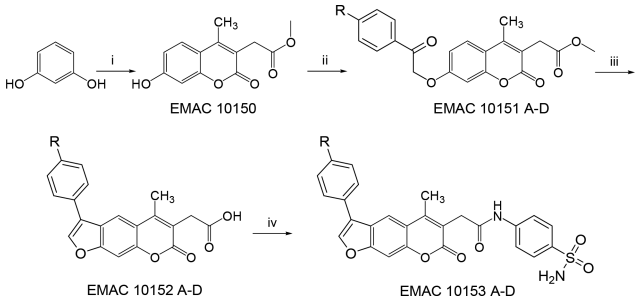
Pursuing on our efforts in the design and synthesis of hCA inhibitors,[31,32] we have designed and synthesized a small library of coumarin (**EMAC10151 A-D**) and psoralen (**EMAC10152 A-D**) derivatives with the aim to target the hCA IX and XII isoforms. To achieve a deeper insight in the inhibitory potential of such derivatives, we have also synthesized **EMAC10152 A-D** and the corresponding benzenesulfonamide hybrids **EMAC10153 A-D**. The new compounds were synthesized by a versatile multistep synthetic approach (Scheme 1). Methyl 2-(7-hydroxy-4-methyl-2-oxo-2*H*-chromen-3-yl)acetate **EMAC10150** was obtained by Pechmann condensation of resorcinol with dimethylacetylsuccinate in 98% sulfuric acid. **EMAC10150** was converted into methyl 2-(4-methyl-2-oxo-7-aryloxy-2*H*-chromen-3-yl)acetate derivatives **EMAC10151 A-D** by Williamson reaction with the appropriate α-haloketone, in the presence of dry acetone and using potassium carbonate to generate the *in situ* alkoxide ion.

By heating **EMAC10151 A-D** in sodium hydroxide 1 M solution, the simultaneous intramolecular condensation and ester saponification took place to give 2-(5-methyl-7-oxo-3-aryl-7*H*-furo[3,2-g]chromen-6-yl)acetic acid **EMAC10152 A-D**.

Scheme 1. Synthetic Pathway to Compounds EMAC10151 A-D, EMAC10152 A-D, and EMAC10153 A-D[a]



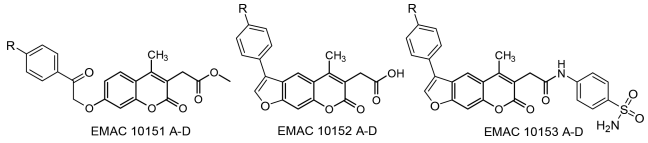
[a]Reagents and conditions (i) dimethylacetylsuccinate, H_2SO_4 98%, RT; (ii) dry acetone, K_2CO_3, α-haloketone, reflux, 1−5 h.; (iii) propan-2-ol, NaOH 1 N, reflux, 4 h; (iv) $SOCl_2$, RT, dry acetone, 4-aminobenzenesulfonamide, dry pyridine.

The formation of the 2*H*-furo[2,3-h]chromen-2-one isomers was not observed, as confirmed by ^{1}H NMR spectroscopy (Figure S1). Benzenesulfonamide hybrids **EMAC10153 A-D** were obtained via acyl chloride formation and subsequent reaction with 4-aminobenzenesulfonamide. All compounds were characterized by means of both analytical and spectroscopic methods (Figures S2−S25) and finally evaluated for the inhibition of four hCA isoforms I, II, IX, and XII (Table 1).

Most **EMAC** compounds possess, although with some distinction, selective activity toward the IX and XII isoforms. However, when the benzenesulfonamide moiety is present, a strong reduction of the selectivity becomes evident. In particular, in the case of compound **EMAC10153 D**, the complete loss of selectivity was observed.

On the contrary, both **EMAC10151 A-D** and **EMAC10152 A-D** are generally potent and selective inhibitors of the tumor associated hCA IX and XII isoforms. Nevertheless, some considerations should be reported. Regarding the methyl 2-(4-methyl-2-oxo-7-aryloxy-2*H*-chromen-3-yl)acetate derivatives, **EMAC10151 A-D**, a different activity profile could be observed, according to the substitution of the phenyl ring. Thus, the introduction of a halogen atom (Cl or F,

Table 1. Inhibition Data (K_i (nM)) Towards hCA I, II, IX, and XII of Compounds EMAC10151 A-D, EMAC10152 A-D, and EMAC10153 A-D



compound EMAC	R	hCAI	hCAII	hCAIX	hCAXII
10151 A	Cl	>10000	>10000	23.6	446.6
10151 B	CH_3	>10000	>10000	122.8	56.6
10151 C	H	>10000	>10000	89.7	72.5
10151 D	F	>10000	>10000	84.7	250.0
10152 A	Cl	>10000	>10000	94.7	9.3
10152 B	CH_3	>10000	>10000	23.0	9.1
10152 C	H	>10000	>10000	17.5	9.4
10152 D	F	>10000	>10000	17.7	7.4
10153 A	Cl	6829.7	55.1	17.8	2.4
10153 B	CH_3	7069.0	560.0	91.6	3.4
10153 C	H	7016.1	46.6	16.5	3.6
10153 D	F	7148.1	79.5	108.4	49.9
AAZ	//	250	12.1	25.8	5.7

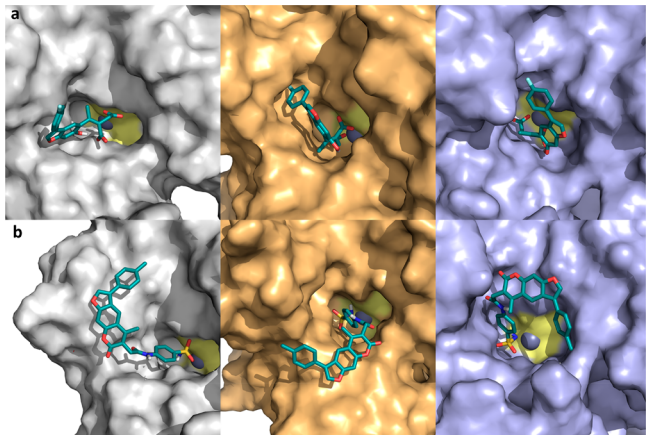


Figure 1. Three-dimensional representation of docking results of (a) **EMAC10152 D** in cyan and (b) **EMAC10153 B** in pink with the three hCA isoforms represented as surface: gray, hCA II; beige, hCA IX; light-blue, hCA XII. The active site is highlighted in pale yellow.

EMAC10151 A and **EMAC10151 D**) in position 4 of the phenyl substituent oriented the activity and selectivity toward the IX isozyme. On the contrary, the unsubstituted phenyl or the 4-CH_3-phenyl moieties led to an increase of the activity toward the hCA XII isoform. Indeed, a reduction of the activity toward hCA IX was observed for compound **EMAC10151 B**. However, although weaker (K_i = 122.8 nM), this derivative exhibited a high selectivity toward the hCA IX and XII isozymes. A similar behavior could be observed for all the psoralen carboxylic acid derivatives **EMAC10152 A-D.** All these compounds were potent and selective tumor associated isoforms IX and XII hCAIs. Their K_i values toward hCA IX and XII ranged from 94.7 to 7.4 nM, while no inhibition could be observed up to 10 000 nM concentration toward the off-target hCA I and hCA II isozymes. In more detail, it could be pointed out that, in the case of hCA IX inhibition, the biological activity is more influenced by the size of the substituent on the phenyl ring rather than by its nature. Thus, the introduction of a chlorine atom (**EMAC10152 A**) led to the less potent

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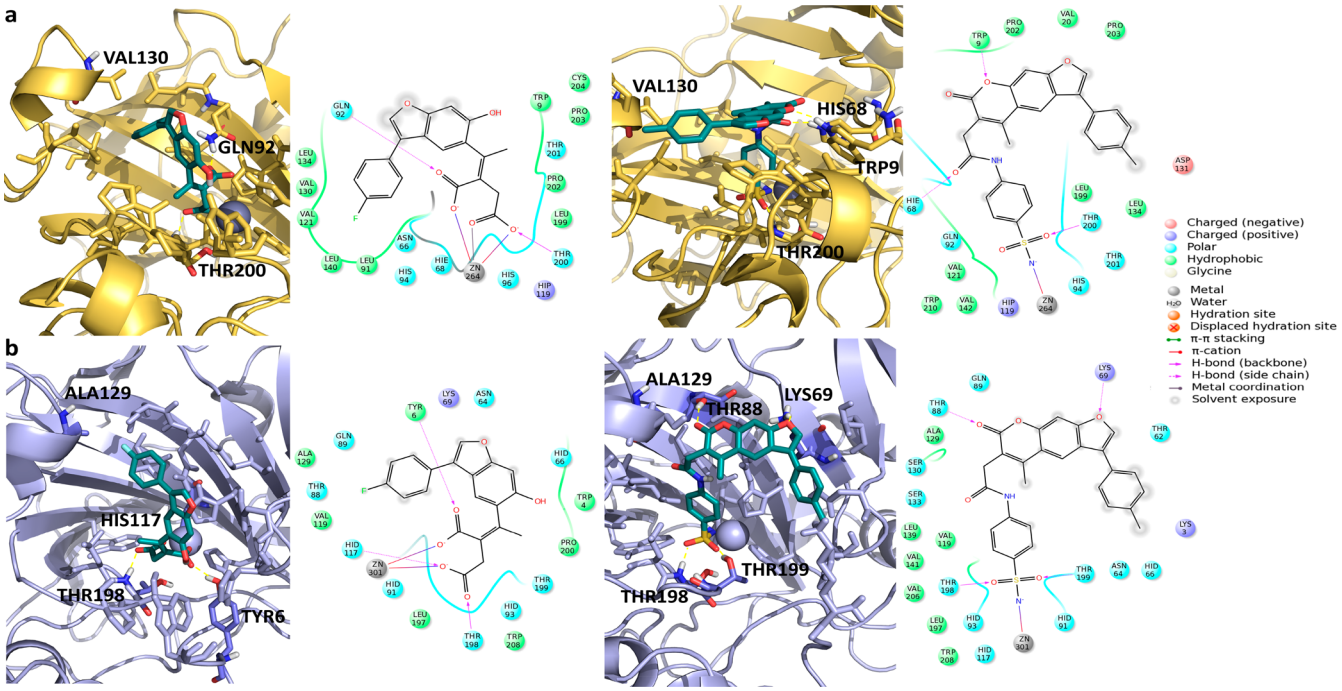

Figure 2. Three-dimensional representation of the putative binding mode obtained by docking experiments of **EMAC10152 D** and **EMAC10153 B** into (a) hCA IX (beige) and (b) hCA XII (light blue), and the relative 2D representation of the complexes stabilizing interactions with the residues of the binding site.

compound within the **10152** series. On the contrary, compounds **EMAC10152 C** and **EMAC10152 D**, bearing an unsubstituted phenyl and a fluoro phenyl moiety, respectively, resulted as the most potent inhibitors. Interestingly, hCA XII is inhibited at low nanomolar concentrations, regardless of the nature of the substitution. As mentioned above, in the case of the psoralen-benzenesulfonamide hybrids (**EMAC10153 A-D**), a potent activity toward the hCA XII was observed for most of the tested derivatives. The inhibition of the XII isozyme could still be observed at concentrations ranging from nanomolar to low nanomolar, but a lower selectivity is generally measured toward the off-target isozyme hCA II with respect to **EMAC10151** and **EMAC10152** series. Only in the case of compound **EMAC10153 B**, bearing a methylphenyl moiety on the furan ring, a good selectivity toward hCA IX and XII versus hCA I and II was found.

Computational methods have been applied in order to explain the selectivity of the synthesized coumarin derivatives toward hCA IX and hCA XII isoforms and their inability to inhibit the II isoform, which, in our intent, represents an off-target. To perform our studies, we chose the two compounds that showed the highest potency and selectivity toward hCA IX and XII: the acid derivative **EMAC10152 D** and the sulfonamide derivative **EMAC10153 B**. Indeed, these compounds have been docked inside the three crystal structures II (PDB 3f8e), IX (PDB 5fl4), and XII (PDB 4ww8).[33,34] The previously validated QMPL[35] protocol was applied.[32] Both coumarin derivatives have been prepared considering the possibility that the coumarin moiety can be hydrolyzed by the Zn^{2+} activated water molecule of the enzyme cavity, which acts as a very potent nucleophile.[33] Therefore, the compounds were ionized at pH 7.4. However, the sulfonamide group was considered both unionized, at pH 7.4, and ionized, taking into account the micro-basic pH inside the binding pocket (Figure S26). All molecules were subjected to conformational analysis, and the global minimum of each was selected for carrying out docking experiments. The putative binding mode suggested by docking simulations well explain the selectivity of our compounds. In fact, both compounds are not able to enter deeply in the active site of hCA II because of the steric hindrance of Phe 131 (Figures 1 and S27). On the contrary, they perfectly fit into the active site of hCA IX and hCA XII, where Phe 131 is substituted by Val 130 and Ala 129, respectively (Figure 1). In particular, the suggested binding mode shows that the coumarin moiety of compound **EMAC10152 D** can reach the catalytic site and be hydrolyzed. Conversely, like most sulfonamide derivatives, **EMAC10153 B** acts by interacting with the sulfonamide moiety, while the coumarin portion, oriented outside the pocket, cannot be hydrolyzed (Figure 1). According to the above suggested mechanism, only the complexes with hydrolyzed **EMAC10152 D** and closed **EMAC10153 B** were subjected to a postdocking procedure based on energy minimization in order to take into account the induced fit phenomena that occur upon ligand binding.[36] The docking results are in agreement with experimental data regarding the isoform selectivity. In fact, EMAC compounds bound to hCA IX and XII showed lower G-score compared to the complex with isoform II (Table S1), indicating a major stability of the complexes with hCA IX and XII in agreement with the experimental results (Table 1).

The analysis of the interaction mode with isoforms IX and XII (Figure 2) shows that **EMAC10152 D** and **EMAC10153 B** are able to establish a wide array of hydrogen bonds with crucial residues of the binding site and several hydrophobic interactions. In more detail, the carboxylic function of **EMAC10152 D** interacts as a bidentate chelator with Zn^{2+}. Conversely, in the case of **EMAC10153 A**, the sulfonamide group plays a key role by interacting with Zn^{2+} and Thr residues, as it has been generally observed for this class of compounds.[37,38]

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Therefore, a putative binding mode seems to confirm the importance of the coumarin moiety in both closed and hydrolyzed conformations, although the different functionalization of EMAC compounds with respect to previously reported coumarins[33] led to a different target recognition that involves the interaction with the zinc ion. Hence, this could represent an innovative mode of action. In addition, it was confirmed that a bulky moiety, linked to the catalytic site binding scaffold, allows selectivity over the off-target isoform II. In fact, in the case of isoform II, the steric hindrance of Phe131 prevents the ligand−target interaction.

According to these results, both psoralen and the hybrid compounds could be considered as promising scaffolds for the design of high selective inhibitors of the hCAs IX and XII isoforms. However, while very high selectivity was always achieved by coumarin (**EMAC10151**) and psoralen (**EMAC10152**) derivatives, the same behavior was not observed in the case of the hybrid compounds **EMAC10153**. Probably, this is mainly due to the presence of the strong Zn^{2+} binder benzenesulfonamide, which, on the one hand, may lead to very potent compounds but, on the other, could affect selectivity.

Conversely, hydrolyzed coumarin **EMAC10151** and psoralen **EMAC10152** most likely act as bidentate Zn^{2+} chelators, while hybrid compounds **EMAC10153** may preferentially act as traditional Zn^{2+} binders. These results were very encouraging and pushed us to further investigate these derivatives in order to identify potential candidates for the treatment of hypoxic tumors.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.8b00170.

Experimental procedures and compounds characterization (PDF)

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

HIF-1α, hypoxia-inducible factor 1-alpha; mTOR, mammalian target of rapamycin; p70S6K, ribosomal protein S6 kinase beta-1; 4E-BP1, eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1; MAPK, mitogen-activated protein kinase; hCA, human carbonic anhydrase; AAZ, acetazolamide

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